CURRENT MICROBIOLOGY Vol. 48 (2004), pp. 262–269
DOI: 10.1007/s00284-003-4186-2

Current Microbiology
An International Journal

AbrB and Spo0E Control the Proper Timing of Sporulation in *Bacillus subtilis*

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Received: 19 July 2003 / Accepted: 13 August 2003

Abstract. We have shown previously that Spo0A∼P-dependent *sinIR* operon expression was substantially down-regulated in *abrB* null mutant backgrounds. In this report, we show that loss of function mutations in *abrB* also cause phosphorelay gene expression to be down regulated. *abrB* null mutations caused diminished vegetative growth-associated sporulation and resulted in a significant reduction in sporulation frequencies at T_{24}. These mutants, however, sporulated at wild-type levels at T_{48}, indicating that sporulation timing was affected. The *rvtA11* mutation in *spo0A*, a deletion mutation in *spo0E*, and a null mutation in *hpr* (*scoC*) rescued sporulation and Spo0A∼P-dependent gene expression in an *abrB* mutant background. These data indicate that AbrB and Spo0E may comprise a checkpoint system that regulates the progression of sporulation, allowing exploration of alternate cell states prior to the irrevocable commitment to sporulation.

In response to nutrient limitations, *Bacillus subtilis* cells cease exponential growth and enter the stationary phase. Depending on the environmental cues present, *B. subtilis* postexponential transition-state regulation can channel a cell toward motility, nutrient scavenging through the production of extracellular enzymes, competence, or sporulation cell fates {for review, see refs. [5, 25]}. One of the early required events for sporulation initiation is the induction of *sinIR* operon transcription from the P1 promoter [3, 7, 20]. Transcription of *sinIR* leads to increased in vivo levels of SinI, product of the first gene in the operon that in turn posttranslationally antagonizes the activity of the SinR sporulation repressor, the product of the second gene in the operon [3]. The decision to sporulate ultimately depends on the activity of two key transcription factors, Spo0A and AbrB. Spo0A acts as a positive regulator of sporulation, and AbrB functions to prevent sporulation {reviewed in [19]}. Consistent with this view, we found *sinIR* expression to be positively regulated by Spo0A [20]. Unexpectedly, we found *sinIR* expression to be substantially diminished in *abrB* null mutant backgrounds [20].

In this report, we show that AbrB interaction with the *sinIR* operon is repressive in nature. We demonstrate that the proper timing and efficiency of sporulation requires functional AbrB. We show that in *abrB* null mutant backgrounds, the expression of *spo0A*, *spo0F*, and *kinA* is down-regulated. This reduction in sporulation gene expression is physiologically correlated with a reduction in the production of endospores. An extragenic suppressor mutation in *spo0A* (*rvtA11*), which bypasses the requirement for the phosphorelay [23], was able to restore sporulation in *abrB* null mutant backgrounds, indicating the in vivo levels of Spo0A∼P may be reduced in these genetic backgrounds. Spo0E protein phosphatase has been shown to inhibit sporulation by inactivating Spo0A∼P, the master regulator of sporulation initiation [14, 17]. *spo0E* expression increased significantly in *abrB* null mutants. A loss of function mutation in *spo0E* concomitantly rescued sporulation and sporulation gene expression in *abrB spo0E* double mutant background. These results suggest that reduced sporula-

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Table 1. Bacterial strains

Strain	Description (relevant genotype)[a]	Source or reference[b]
1A180	*scoC (hpr-16)*	BGSC
RS1000	*168*	This laboratory
RS1001	*metC2 lys-1*	This laboratory
RS1004	*spo0A12*	These laboratories
EE1005	*abrB::Tn917*	This laboratory
SWV119	*abrB::Tet trpC2 phe-1*	[20]
SWV185	*abrB::Tet trpC2 phe-1 spo0E::lacZ*	[20]
RS5101	*rvtA11*	This laboratory
SS11	*metC2 lys-1 sinI::lacZ*	This laboratory
SS13	*sinI::lacZ*	This laboratory
SS15	*spo0A12 sinI::lacZ*	SS13→ RS1004 (Cm^r selection)
SS47	*spo0A::lacZ*	This laboratory
SS48	*spo0F::lacZ*	This laboratory
SS54	*kinA::lacZ*	This laboratory
SS27	*rvtA11 sinI::lacZ*	SS13→ RS5101 (Cm^r selection)
SS29	*hpr-16 sinI::lacZ*	SS13→ 1A180 (Cm^r selection)
SS33	*abrB::Tn917 sinI::lacZ*	SS13→ EE1005 (Cm^r selection)
SS35	*abrB::Tet sinI::lacZ*	SS13→ SS43 (Cm^r selection)
SS36	*abrB::Tn917 hpr-16 sinI::lacZ*	EE1005→ SS29 (MLS^r selection)
SS37	*abrB::Tn917 rvtA11 sinI::lacZ*	EE1005→ SS27 (MLS^r selection)
SS38	*abrB::Tet spo0A12 sinI::lacZ*	SS43→ SS15 (Tet^r selection)
SS41	*spo0E::lacZ*	SWV185→ RS1000 (Cm^r selection)
SS42	*abrB::Tn917 spo0E::lacZ*	SWV185→ EE1005 (Cm^r selection)
SS43	*abrB::Tet*	SWV119→ RS1000 (Tet^r selection)
SS44	*abrB::Tet spo0E::lacZ*	SWV185→ RS1000 (Cm^r selection & congression)
SS50	*abrB::Tn917 spo0A::lacZ*	EE1005→ SS47 (MLS^r selection)
SS51	*abrB::Tet spo0A::lacZ*	SS43→ SS47 (Tet^r selection)
SS52	*abrB::Tet hpr-16 spo0A::lacZ*	SS51→ 1A180 (Tet^r selection & congression)
SS53	*abrB::Tn917 spo0F::lacZ*	EE1005→ SS48 (MLS^r selection)
SS55	*abrB::Tet spo0F::lacZ*	SS43→ SS48 (Tet^r selection)
SS56	*abrB::Tet hpr-16 spo0F::lacZ*	SS55→ 1A180 (Tet^r selection & congression)
SS59	*abrB::Tn917 kinA::lacZ*	EE1005→ SS54 (MLS^r selection)
SS60	*abrB::Tet kinA::lacZ*	SS43→ SS54 (Tet^r selection)
SS61	*abrB::Tet hpr-16 kinA::lacZ*	SS60→ 1A180 (Tet^r selection, Cm^r screening)
SS3050	*abrB::Tet spo0E::Em sinI::LacZ*	Linearized pSS3000→ SS35 (Em^r selection)

[a] For clarity, the auxotrophic genotypes of some strains have been omitted.
[b] BGSC, *Bacillus* Genetics Stock Center.

tion gene expression and sporulation in *abrB* mutant backgrounds may be due to premature accumulation of Spo0E.

Materials and Methods

Bacterial strains. The strains used, their genotypes, and their sources are listed in Table 1.

Plasmid and β-galactosidase synthesis by *B. subtilis lacZ* fusion strains. The following *lacZ* fusions were used in these studies: *spo0A::lacZ* is a transcriptional fusion ectopically introduced into the *amyE* locus and containing both the vegetative and sporulation promoters as described [9]. *spo0F::lacZ* and *kinA::lacZ* [1, 10] are translational fusions and were introduced into the *amyE* locus as described [11]. *spo0E::lacZ* is an ectopic transcriptional fusion described previously [17]. This fusion was ectopically introduced into the *amyE* locus by selecting for the vector-associated chloramphenicol resistance gene [11, 24]. The expression of *lacZ* fusions was determined as described [4]. Specific activity is expressed as nanomoles of *o*-nitrophenyl hydrolyzed per milligram of cellular protein per minute.

Cell growth, induction of sporulation, and sporulation quantitation. Cell growth, induction of sporulation in 2 × SG sporulation medium and sporulation quantitation were performed as described [23].

PCR analyses of the *abrB* locus. To confirm the presence of *abrB::Tet* and *abrB::Tn917* mutations, *abrB* locus was amplified by PCR in a robocycler (Stratagene) with the XL PCR kit (Perkin Elmer) and the following program: 35 cycles of 94°C (30 s), 46°C (1 min), and 68°C (5 min). The primers used were as follows: **abrB-Fwd-3** (−178 to −159 with respect to the start codon ATG, with A serving as position +1): 5′-CTGTTATTTCGGTAGTTTC-3′ and **abrB-Rev-3** (+195 to +176 with respect to the start codon ATG, with A serving as position +1): 5′-ATCATCAGAAACTTCACCAG-3′.

We expected fragments of approximately 350 bp, 3500 bp, and

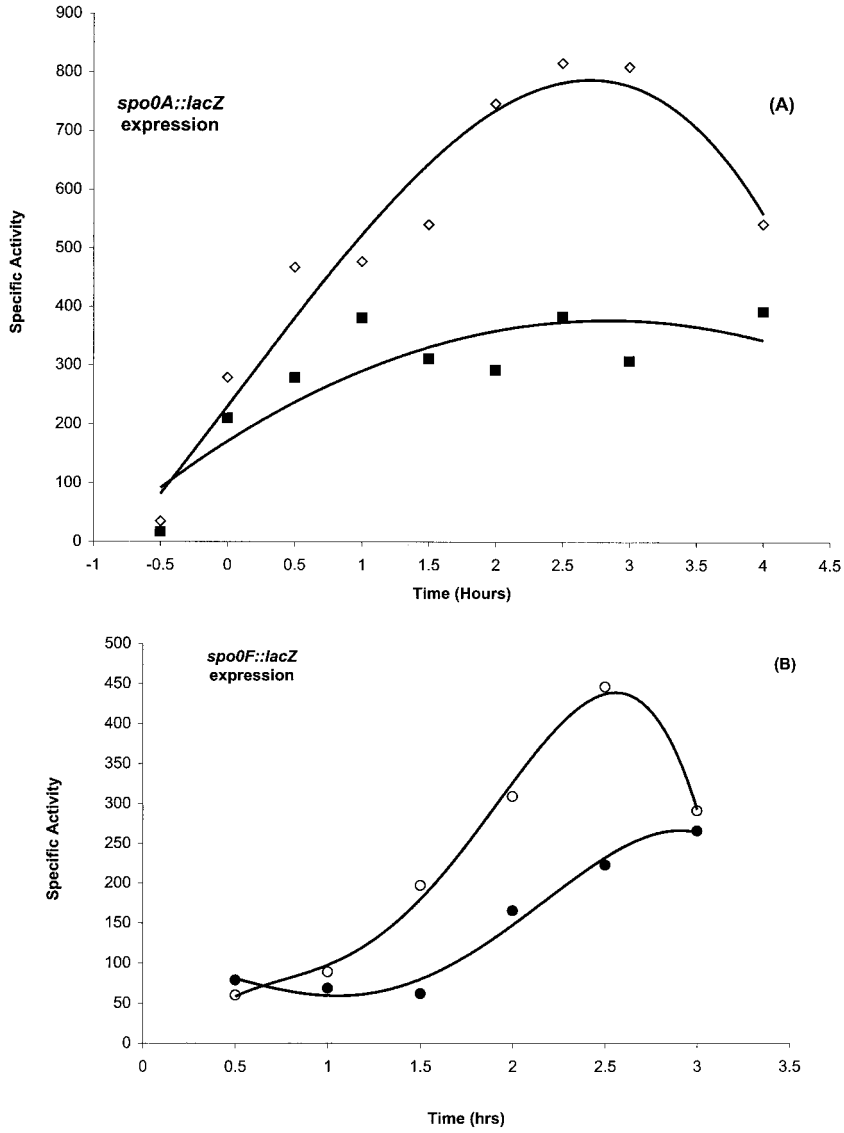

Fig. 1. The negative effect of an *abrB* null mutation on the expression of components of the phosphorelay. The indicated strains were grown in 2 × SG sporulation medium and were analyzed as described previously [4]. T_o denotes the end of exponential growth. A) *spo0A::lacZ* expression in SS47 (wild-type) (◇) and SS51 (*abrB::Tet*) (■) strains. B) *spo0F::lacZ* expression in SS48 (wild-type) (○) and SS53 (*abrB::Tet*) (●) strains.

5400 bp, for wild-type *abrB*, *abrB::Tet* mutant [26], and *abrB::Tn917* mutant [28], respectively.

Results

Post-exponential induction of phosphorelay gene expression is dependent on functional AbrB. Previously, we have demonstrated that *sinIR* expression is dependent upon activation of Spo0A by phosphorylation through the phosphorelay [20]. We have shown that null mutations in *spo0A*, *spo0F*, and *kinA*, components of phosphorelay, abolished *sinIR* expression. We have also found that catabolite repression of sporulation occurs as a consequence of reduction in expression of the phosphorelay components, *spo0A*, *spo0F*, and *kinA* [21, 22]. We have shown that reduction in the phosphorelay gene expression leads to reduced Spo0A∼P and Spo0A∼P-dependent gene expression, including *sinIR* expression. We examined the effects of *abrB* loss of function mutations on the expression of *spo0A*, *spo0F*, and *kinA*, components of the phosphorelay, and found these genes also to be substantially down-regulated (Fig. 1 and data not shown), suggesting that the decline in *sinI* expression in *abrB* mutant backgrounds may be an indirect effect of the reduction in Spo0A∼P.

A decline in the expression of phosphorelay components would also be expected to adversely affect sporulation. Surprisingly, we did not find any published data regarding *abrB* null mutant sporulation levels. Upon examination, we found that null mutations in *abrB* reduced vegetative growth-associated sporulation at least

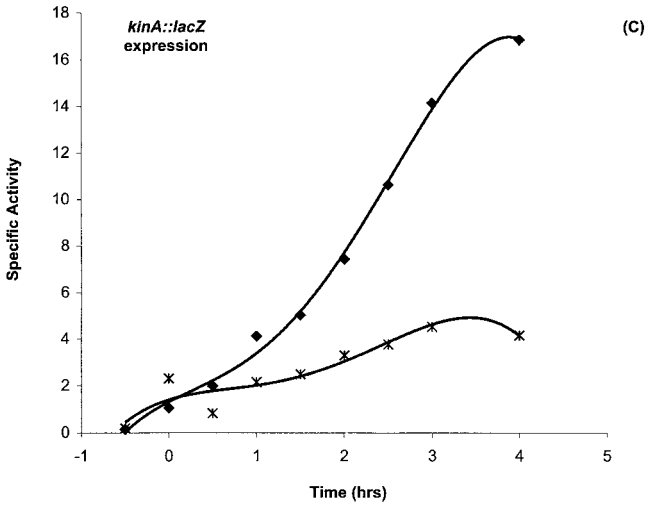

Fig. 1. C) *kinA::lacZ* expression in SS54 (wild-type) (◆) and SS60 (*abrB::Tet*) (✳) strains.

Table 2. Sporulation frequency of wild-type and mutant strains

Strain/relevant genotype	Cell viable count[a] (*cfu*)/mL	Spore count[b] (*cfu*)/mL	Percent sporulation[c]
168 (wild-type)	5.7×10^8	3.0×10^8	53
abrB::Tet	5.7×10^8	4.4×10^7	7.8
abrB::Tn917	4.7×10^8	2.8×10^7	5.9
abrB::Tet scoC (hpr-16)	4.5×10^8	2.0×10^8	44
abrB::Tet rvtA11	8.5×10^8	4.8×10^8	56
abrB::Tet spo0E::Em	8.1×10^8	6.0×10^8	74

[a] Cells were grown in Schaeffer sporulation medium. Total viable counts were determined as c.f.u. at T_3 and T_{24}.
[b] Spore count was determined at T_{24} as c.f.u. after chloroform treatment.
[c] The sporulation frequency was determined by dividing the spore count by the highest viable count.

two log orders below those of wild-type cells (Table 3). These mutants also sporulated less efficiently (6–9%) compared with wild type (>50%) at T_{24} (Table 2). However, sporulation approached wild-type levels (36–41%) at T_{48} (Table 3), 48 h after the onset of sporulation, indicating that sporulation timing was affected in these mutant backgrounds.

Loss of function mutations in *abrB* affects in vivo Spo0A~P levels by premature accumulation of Spo0E protein phosphatase. The *rvtA11* mutation in *spo0A* is an altered-function mutation in *spo0A* that renders its activation independent of the phosphorelay [23]. The *rvtA11* mutation increased *sinI::lacZ* expression in an *abrB* mutant background (Fig. 2D). We have also demonstrated that conditions which lead to reduced in vivo Spo0A~P levels and sporulation can be suppressed by null mutations in *hpr* (*scoC4*) [21, 22]. A loss of function mutation in *hpr* (*scoC*) also restored the expression of *sinI* and *spo* genes in an *abrB* mutant background (Figs. 2A–C; data not shown). Both *scoC* (*hpr-16*) and *rvtA11* mutations also suppressed the *abrB* delayed-sporulation phenotype (Table 2), suggesting that in vivo Spo0A~P levels may be reduced in these mutant backgrounds. Down-regulation of sporulation gene expression in *abrB* mutants (Fig. 1 and [20]) could be due to AbrB functioning as an activator of these genes or as a repressor of another gene(s) whose product directly or indirectly inhibits Spo0A~P-dependent gene expression. AbrB functioning as an activator of sporulation gene expression is neither consistent with its temporal expression pattern, as *abrB* expression is at its minimum when the expression of phosphorelay genes and *sinI* peaks [26, 26], nor with the well-documented function of AbrB as a transition state repressor [6, 13, 18, 29]. On the other hand, if one or more proteins, whose function is to prevent or delay sporulation, were under negative control of AbrB, then decreased levels of AbrB could lead to increased expression of these genes. This mechanism would act as a checkpoint to prevent premature entry into sporulation. Spo0E protein phosphatase is an example of one such gene. Spo0E protein phosphatase inhibits sporulation by removing the phosphate group from Spo0A~P [14, 17]. The decline in the expression of *sinIR* in an *abrB* mutant background could be due to a Spo0E-mediated decrease in Spo0A~P concentration. Consistent with a previous report [17], we found that *spo0E* expression increased three- to fourfold in *abrB* null mutant backgrounds (Fig. 2D). A loss of function mutation in *spo0E* restored *sinI* expression (Fig. 2E) and sporulation (Table 2) in an *abrB* mutant background.

Although these results strongly support the notion that the adverse effect of *abrB* null mutations on *sinI*

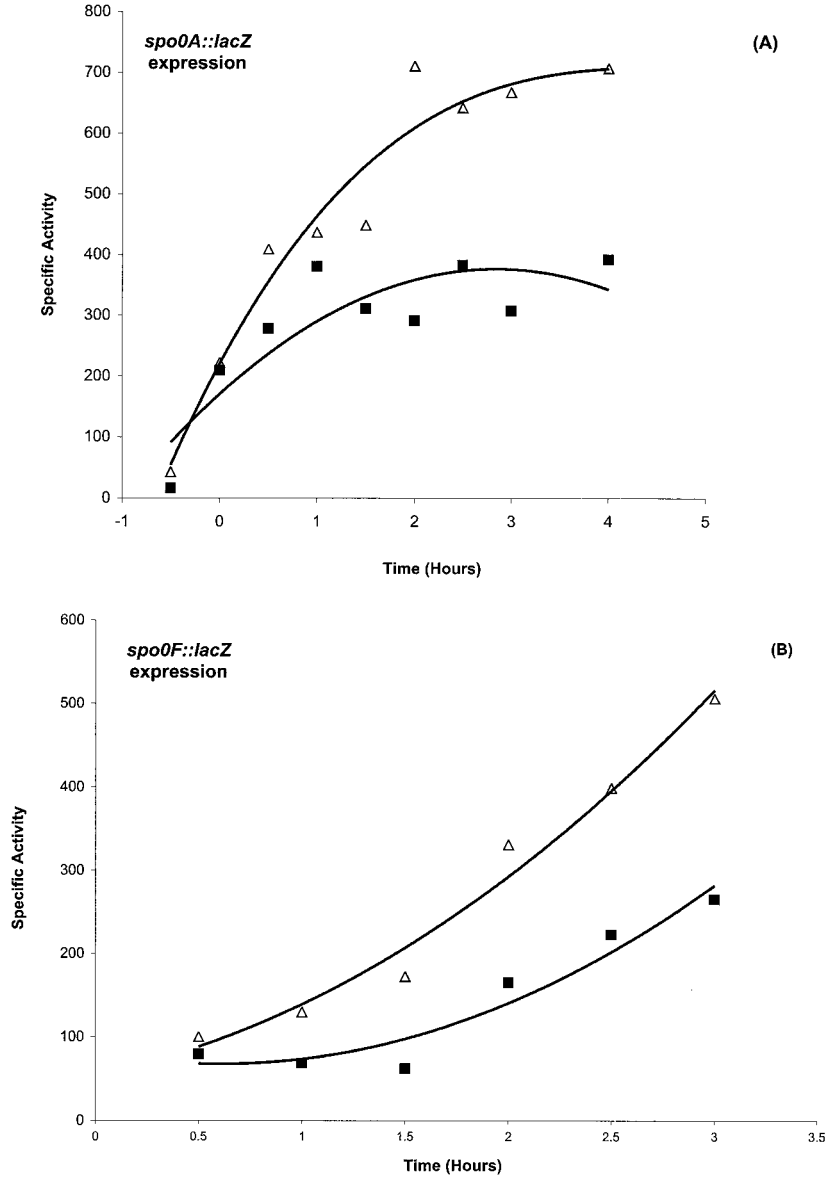

Fig. 2. The negative effect of an *abrB* null mutation on Spo0A∼P-dependent gene expression is relieved by a loss of function mutation in *scoC (hpr-16)*, an altered function mutation in *spo0A* (*rvtA11*), and a deletion in *spo0E*. The indicated strains were grown in 2 × SG sporulation medium and analyzed as described previously [4]. T_0 denotes the end of exponential growth. (A) *spo0A::lacZ* expression in SS51 (*abrB::Tet*) (■) and SS52 (*abrB::Tet hpr-16*) (△) strains. (B) *spo0F::lacZ* expression in SS53 (*abrB::Tet*) (■) and SS55 (*abrB::Tet hpr-16*) (△) strains.

expression may be an indirect consequence of increased *spo0E* expression in these mutant backgrounds, we wished to examine the nature of AbrB binding near to the P1 promoter of *sinIR* operon [20]. We reasoned that in the case of a *spo0A* mutant where there is no Spo0A∼P, Spo0E and other phosphatases that target Spo0A∼P would be predicted not to affect regulation of *sinI* expression. We investigated the regulation of *sinI* expression in *abrB spo0A* double mutants and found it to be consistently elevated in *abrB spo0A* double mutant constructs when compared to its expression in a *spo0A* single mutant, indicating that AbrB control of *sinI* is repressive in nature (Fig. 3).

Discussion

Postexponential induction of *sinI* expression is positively regulated by phosphorylated Spo0A [20] and is required for inactivation of the SinR transition state regulator [8, 12]. *sinI* expression was found to be substantially down regulated in *abrB* null mutant backgrounds [20]. The *rvtA11* mutation, which bypasses the requirement for the phosphorelay [23] in some *spo0* mutants, increased *sinI::lacZ* expression above the wild-type level and restored sporulation (Fig. 2E, Table 2) in an *abrB* mutant background. These results suggest that in vivo Spo0A∼P levels may be adversely affected in these genetic backgrounds. A loss of function mutation in *scoC(hpr-16)*

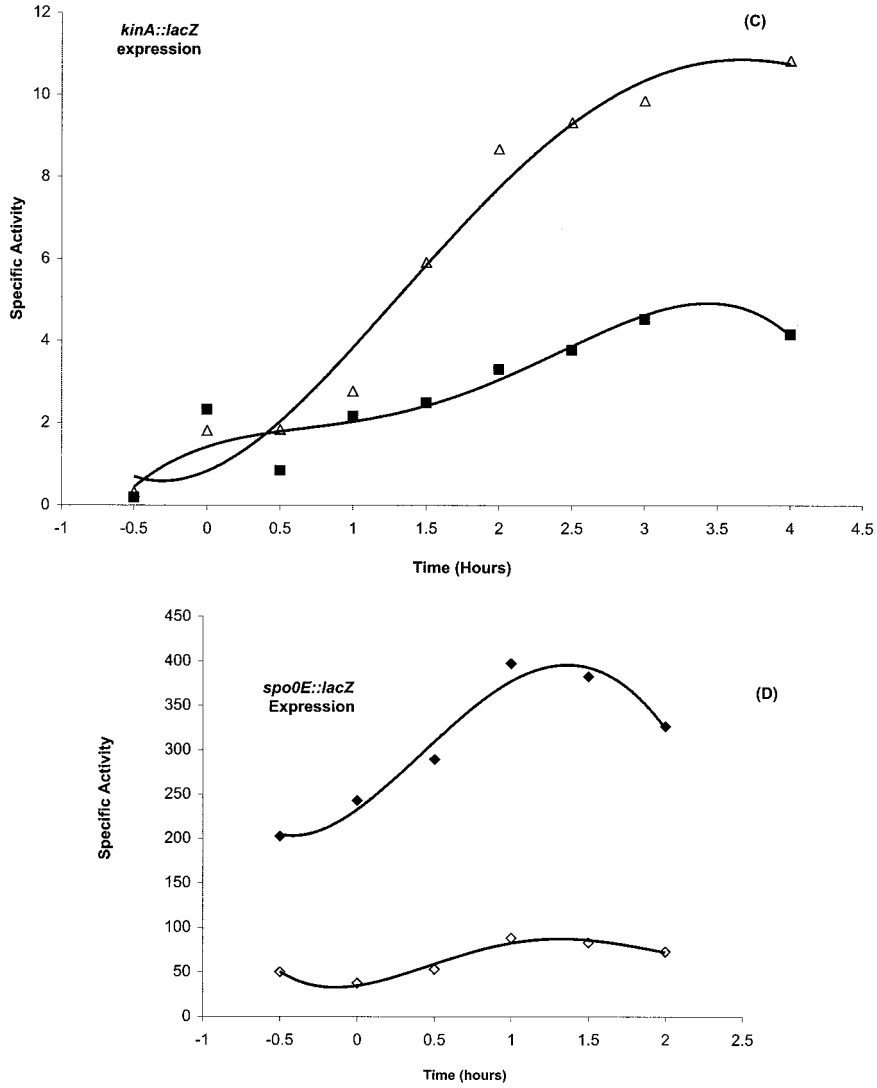

Fig. 2. (C) *kinA::lacZ* expression in SS60 (*abrB::Tet*) (■), and SS61 (*abrB::Tet hpr-16*) (△) strains. (D) *spo0E::lacZ* expression in SS41 (wild-type) (◇) and SS44 (*abrB::Tet spo0E::lacZ*) (◆) strains.

Table 3. Sporulation is delayed in *abrB* mutant strains

Strain/relevant genotype	*168* (wild-type)	*abrB::Tet*	*abrB::Tn917*
Viable count[a] (*cfu*/mL) (T_0)	9.0×10^7	2.5×10^7	ND*
Spore count[b] (*cfu*/mL) (T_0)	2.1×10^5	$<10^3$	ND*
Percent spor.[c] (*cfu*/mL) (T_0)	0.2%	<0.004%	ND*
Viable count[a] (*cfu*/mL) (T_{48})	6.8×10^8	8.9×10^8	6.3×10^8
Spore count[b] (*cfu*/mL) (T_{48})	4.0×10^8	3.2×10^8	2.6×10^8
Percent spor.[c] (*cfu*/mL) (T_{48})	59%	36%	41%

[a] Cells were grown in Schaeffer sporulation medium. Total viable counts were determined as *cfu* (colony forming units) at T_0 and T_{48}.
[b] Spore count was determined at T_0 and T_{48} as *cfu* after chloroform treatment.
[c] The sporulation frequency was determined by dividing the spore count by the highest viable count.
*ND, not determined.

also restored the expression of *sinI::lacZ* and sporulation in an *abrB* mutant background (Fig. 2, Table 2), supporting the notion that ScoC may affect phosphorylation or expression of Spo0A [21, 22]. The decline in *sinI* gene expression is likely to be caused by elevated levels of the phosphoprotein phosphatase, Spo0E (Fig. 2D). Spo0E protein phosphatase has been shown to antagonize the action of Spo0A∼P [14, 17]. Lowering Spo0A∼P levels as a consequence of elevated Spo0E protein phosphatase in *abrB* mutant backgrounds would be expected to adversely affect the expression of genes, such as *spo0A*, *spo0F*, and *kinA*, whose expression is dependent upon Spo0A∼P [2, 15] as well as the timing or extent of sporulation. The finding that expressions of *spo0A*-, *spo0F*-, and *kinA::lacZ* fusions were significantly downregulated in *abrB* mutant backgrounds (Fig. 1) supports this notion. Moreover, vegetative growth-associated

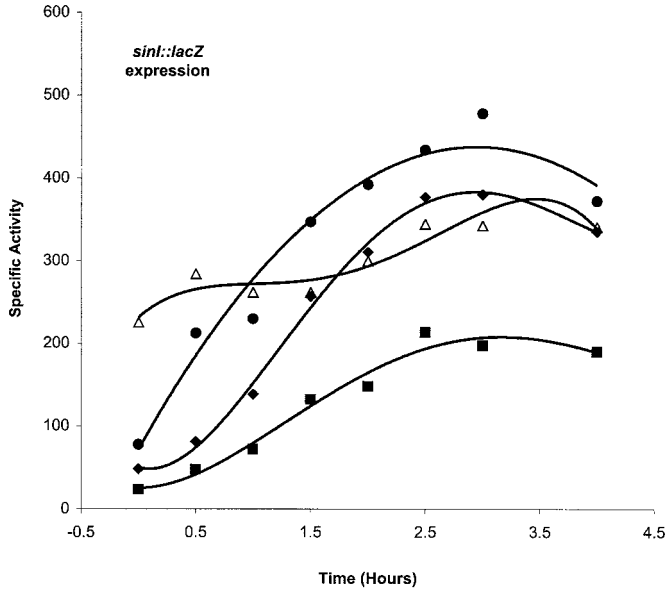

Fig. 2. (E) *sinI::lacZ* expression in SS13 (wild-type) (◆), SS35 (*abrB::Tet*) (■), SS27 (*abrB::Tet rvtA11*) (●), and SS3050 (*abrB::Tet spo0E::Em*) (△) strains.

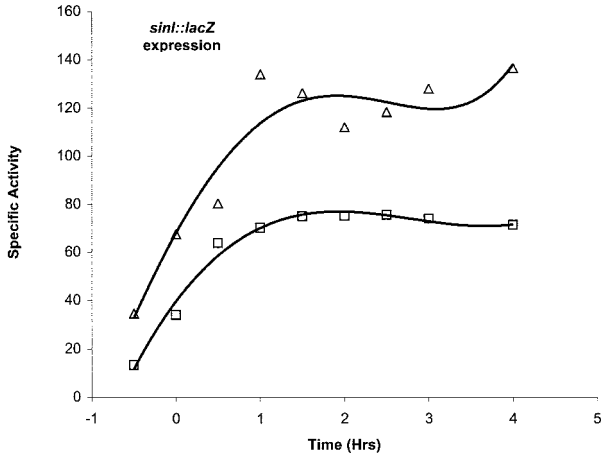

Fig. 3. AbrB is an inhibitor of *sinIR* operon. The indicated strains were grown in 2 × SG sporulation medium and were analyzed as described previously [4]. T_o denotes the end of exponential growth. *sinI::lacZ* expression in SS15 (*spo0A12*) (□) and SS38 (*spo0A12 abrB::Tet*) (△) strains.

sporulation, determined at T_0, was reduced more than two logs in an *abrB* mutant background (Table 3). Sporulation frequencies in these *abrB* mutants remained appreciably lower than the wild-type at T_{24} (6–9% compared with > 50%, Table 2), but approached the wild-type levels at T_{48} (36–41%), indicating that sporulation timing was affected. In addition, a deletion in *spo0E* restored *sinI::lacZ* expression (Fig. 2E) as well as sporulation in an *abrB* mutant background (Table 2).

The data presented here for the first time demonstrate a positive role for AbrB in sporulation. These results suggest that AbrB and Spo0E may participate in a checkpoint mechanism designed to prevent premature commitment to sporulation. At the end of vegetative growth, the initial activation of Spo0A through the phosphorelay results in reduced expression from the *abrB* promoter owing to Spo0A~P inhibition [18]. As a consequence of reduced AbrB inhibition, *spo0E* expression increased (Fig. 2D, [17]) causing a reduction in the rate of Spo0A~P accumulation [14, 17]. This timing delay mechanism allows the exploration of alternate cell states, such as motility, production of extracellular hydrolytic enzymes and antibiotics, and competence (reviewed in [5]) prior to committing to sporulation.

ACKNOWLEDGMENTS

We thank the members of Leighton lab for their helpful comments. This research has been supported by grants from the U.S. Army Corps of Engineers Contract DACA399520005 and DARPA.

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